Quantification of Synthetic DNA Aptamers in Plasma with qPCR: Feasibility & Method Development

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Aptamers as Therapeutics

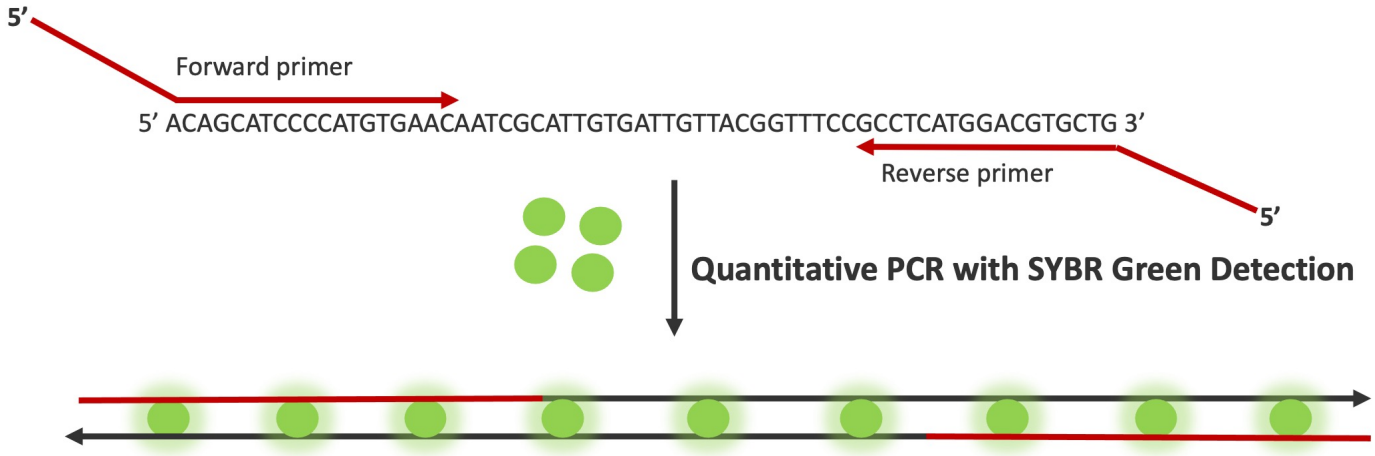
- ssDNA or ssRNA 20-100 nucleotides in length
- Highly folded tertiary structure
- Bind to target molecules with specificity and sensitivity similar to antibodies
  - “Chemical antibodies”
- Advantage over antibodies include:
  - Small size
  - Flexible with rapid synthesis
  - Lower immunogenicity
- Targets include small molecules, metal ions, proteins, viruses, and whole cells
- Aptamer therapeutics can have differing functions
  - Antagonist for blocking disease targets (i.e. receptor-ligand interactions)
  - Agonist to activate targets
  - Cell-specific aptamers to serve as carrier for therapeutic agents

OBJECTIVE

Determine the feasibility of development of a PCR-based method to detect and quantify synthetic DNA aptamers of 40 to 65 nucleotides in length in canine plasma

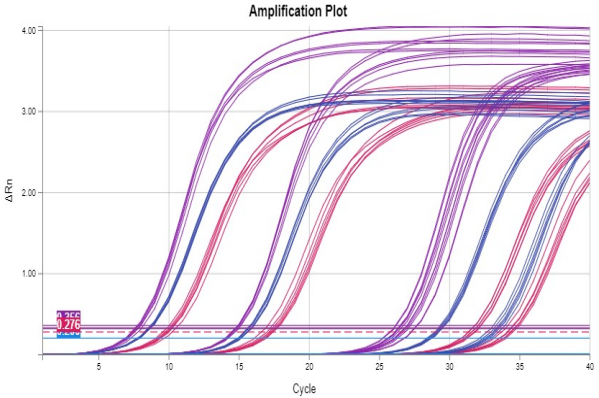
General Approach

Detection of synthetic single-stranded, short DNA aptamers (six) of 39 to 64 nucleotides via QPCR using primers with 5’ extensions on the QuantStudio 7 Pro with SYBR green detection (double-stranded DNA intercalating dye).

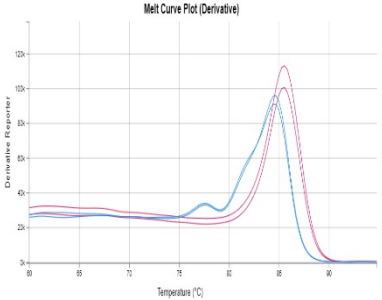


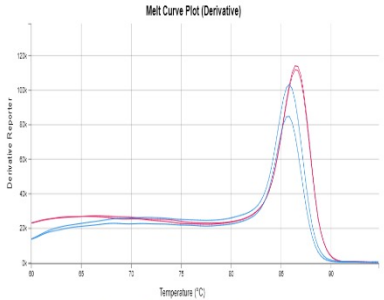
Preliminary Feasibility of the Approach

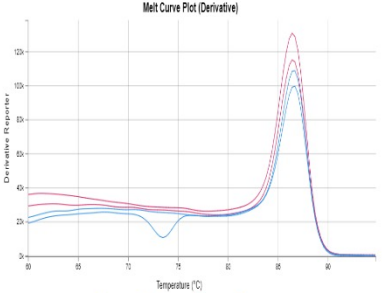
- Four aptamers
  - 39 nt
  - 40 nt
  - 50 nt
  - 51 nt
- Three concentrations and no template control (NTC)
  - 10^8 copies/µl
  - 10^6 copies/µl
  - 10^2 copies/µl
- Three annealing temperatures
  - 58°C
  - 60°C
  - 62°C
- Vendor recommended primer concentrations and cycling conditions
- 40 cycles followed by melt curve



- Three out of four synthetic aptamers successfully amplified at all conditions tested.
  - No signal with 51 nt aptamer at any concentration of input or condition
- Signal in NTC
  - Melt curve indicative of primer-dimer
- Approach feasible with optimization

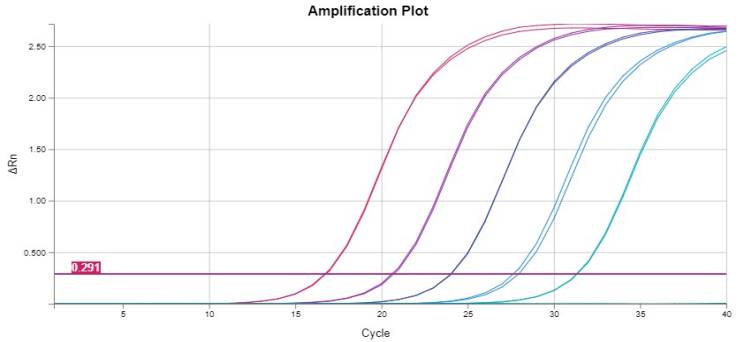


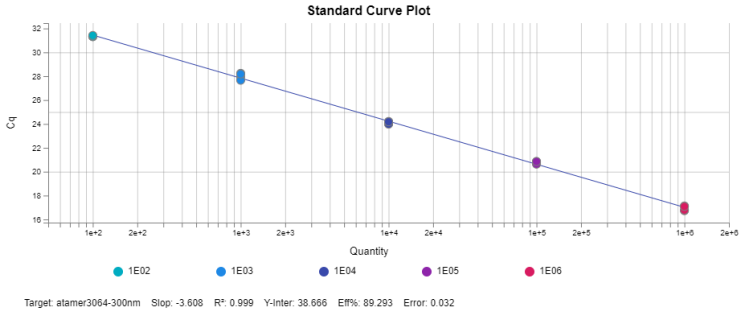




Optimization of Amplification of 40 nt aptamer

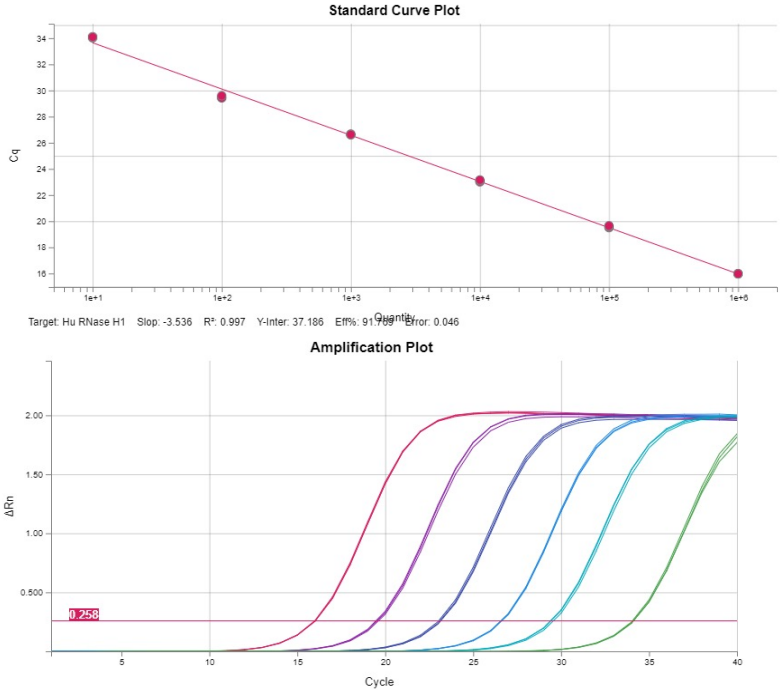
- Published aptamer and primer set for SYBR green detection (Nucleic Acid Therapeutics 25(1):11-19 (2015)
- Temperature gradient (58-65ºC) to identify optimal annealing temperature
- Primer titration (100 nM-400 nM) for optimal primer concentrations
- 40 cycles of amplification followed by melt curve analysis
- Optimized annealing temperature and primer concentrations successfully eliminated signal in NTC
- Preliminary sensitivity 500 copies/reaction





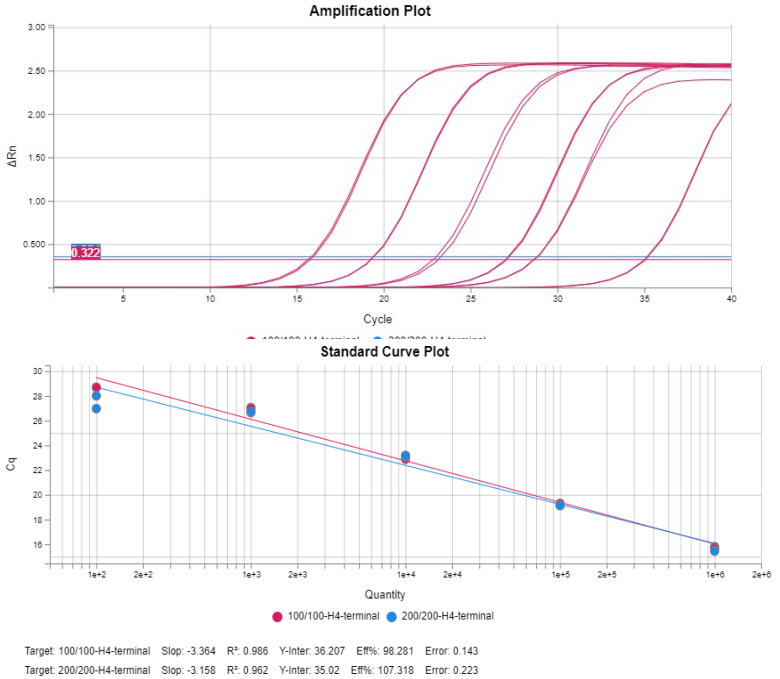
Quantification of 39 nt aptamer

- Reaction condition optimization
  - Temperature gradient
  - Primer titration
- Quantification down to 50 copies/reaction with Relative error <25%
- Signal in no template control (NTC) wells with C_t>35 cycles
- Additional assay optimization to reduce or eliminate background signal
- Concurrent re-design of primers with more favorable heterodimer formation properties



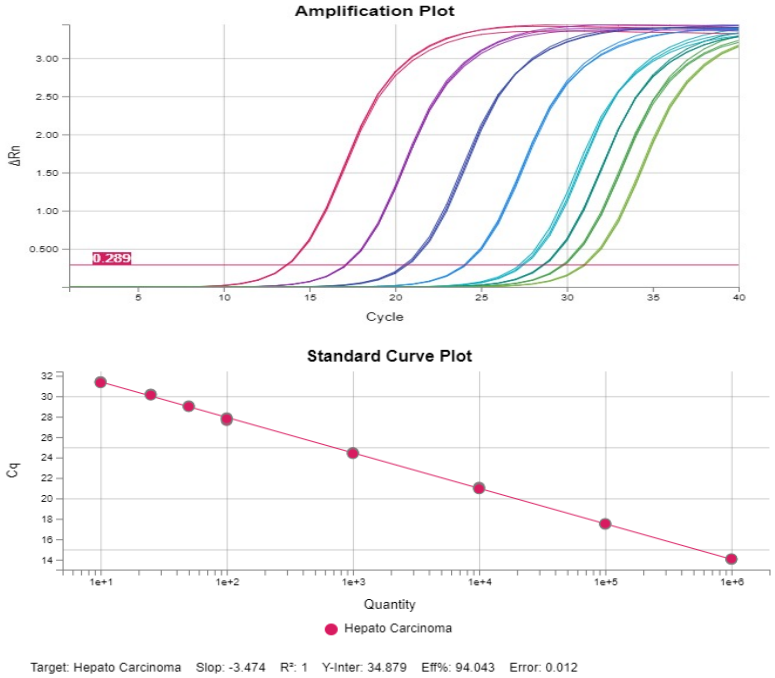
Quantification of 50 and 51 nt aptamers

- Reaction condition optimization
  - Temperature gradient
  - Primer titration
- Aptamer of 51 nucleotides did not amplify under any conditions
- Results of 50 nucleotide aptamer:
  - Consistent signal in NTC wells with C_t values between 29 and 33 cycles
  - Background amplification interfering with assay that is shifting signal in wells with 100 copies/µl
- Re-design primers for both aptamers with more favorable heterodimer formation properties



Quantification of 63 nt Aptamer

- Reaction condition optimization
  - Temperature gradient
  - Primer titration
- Linear quantification down to 50 copies/reaction
  - Relative error <15%
  - Total error <25%
- Optimized assay eliminated all background signal in NTC
- Optimized reaction efficiency 94%



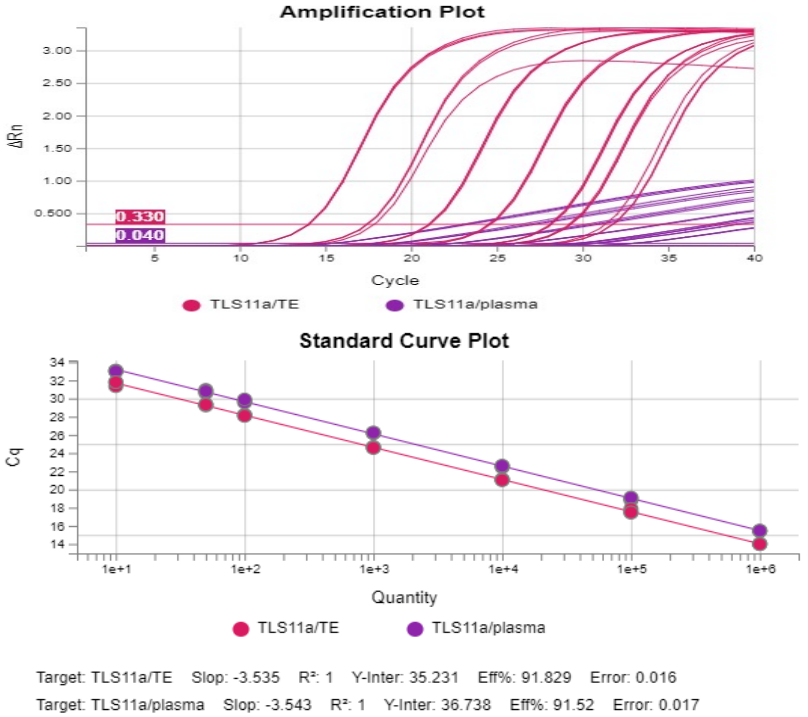
Aptamer Recovery from Canine Plasma

- Canine plasma spiked with 63 nt aptamer
  - 2000 copies/mL
  - 10000 copies/mL
  - 50000 copies/mL
  - Negative control
- Plasma (1 mL) processed with QIAamp Circulating nucleic acid kit using protocol for purification of microRNA
- Elution in 50 µl buffer
- Performed QPCR using 5 µl of eluted DNA
- Recovery of aptamer low with QIAamp protocol (<30%)
- Can aptamer be detected in plasma without purification?

	50000 copies/ml	10000 copies/ml	2000 copies/mL
Expected copies/µl for 100% recovery	1000 copies/µl	200 copies/µl	40 copies/µl
Actual	298 ± 2.63 copies/µl	36.3 ± 0.62 copies/µl	10.9 ± 1.55 copies/µl
% Recovery	29.8%	18.2%	27.3%

Direct detection of 63 nt aptamer in plasma

- Dilution of 63 nucleotide aptamer in 100% plasma
- Used 5 µl per reaction for final plasma concentrations of 25%
- Input of sample in 100% plasma significantly effects amplification
- Y-intercept shifted by 1.5 C_t
- Slopes of standard curves remain similar
- High total error observed for reactions with plasma concentrations of 25%

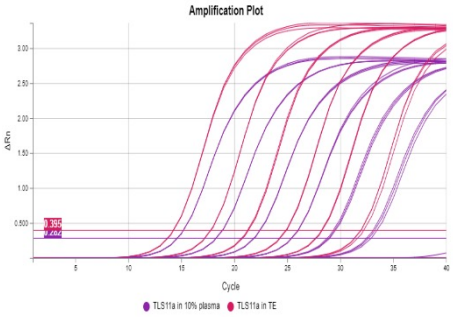


Direct detection of 63 nt aptamer in diluted plasma

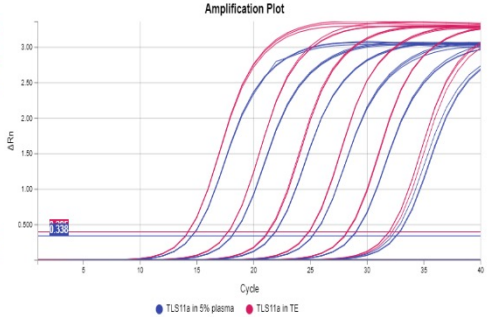
- Serial dilutions of aptamer (63 nt) prepared in 5% or 10% plasma.
- Final plasma concentrations in QPCR reactions are 1.25% and 2.5%
- Slope of standard curves not affected.
- Dose response shift in y-intercept less that one Ct between aptamer dilutions prepared in TE versus diluted plasma
- Total error (%CV + %RE) increases at lower template concentrations with increasing plasma concentration
  - Total error ~30% at 50 copies input in plasma versus total error <10% at 500 copies input in plasma

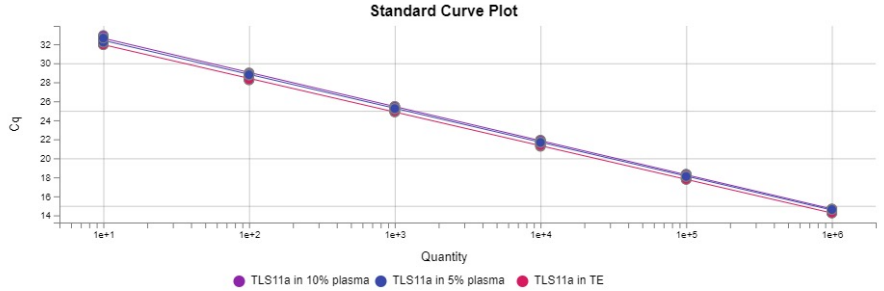
Input	TE	1.25% Plasma	2.5% Plasma
5000000	2.423	3.854	1.366
500000	1.921	1.649	2.452
50000	3.818	1.508	3.965
5000	2.144	5.006	2.253
500	9.211	6.942	9.308
50	10.676	27.008	30.211

TE (red) vs 10% Plasma (purple)



TE (red) vs 5% Plasma (blue)

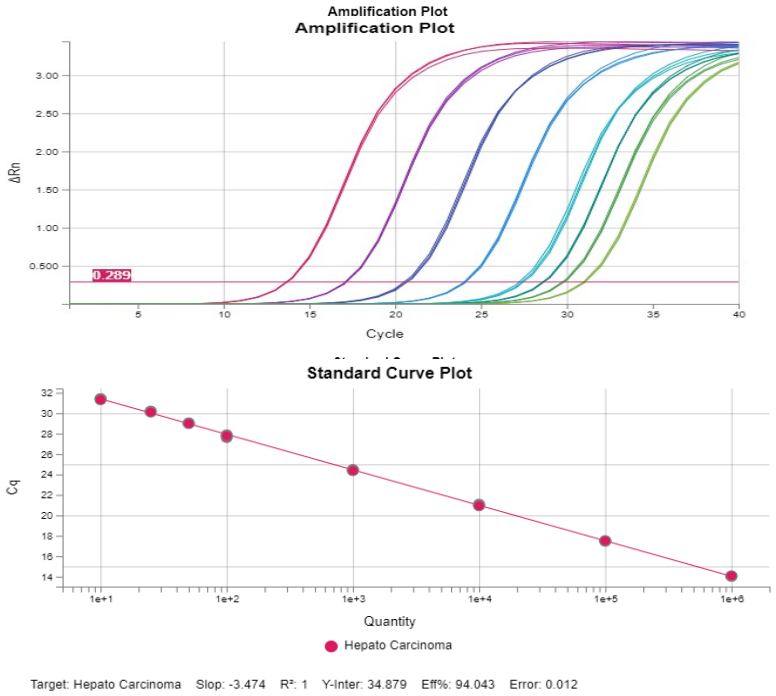




Target: TLS11a in TE Slop: -3.542 R²: 1 Y-Inter: 35.498 Eff%: 91.575 Error: 0.013
Target: TLS11a in 10% plasma Slop: -3.59 R²: 1 Y-Inter: 36.22 Eff%: 89.916 Error: 0.015
Target: TLS11a in 5% plasma Slop: -3.569 R²: 1 Y-Inter: 35.974 Eff%: 90.647 Error: 0.014

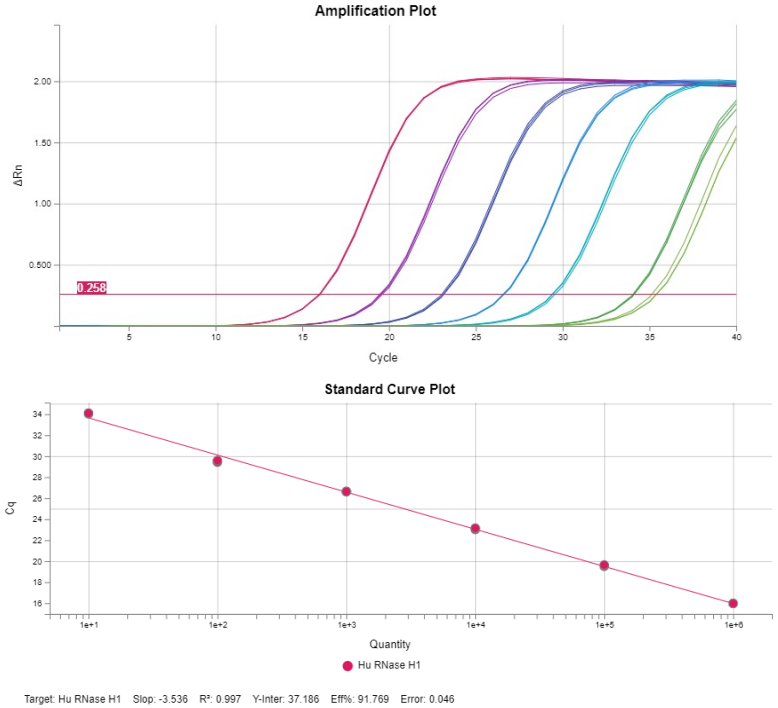
Optimized detection of 64 nt aptamer in diluted plasma

- Assay optimized as described before
  - Primer concentration
  - Annealing temperature
- Serial dilution of 64 nucleotide aptamer in 5% plasma
- Used 5 µl per reaction for final plasma concentrations of 1.25%
- Linear quantification down to 500 copies/reaction
  - Detection of amplification of 250 and 50 copies/reaction
- Slopes of standard curves remain similar to aptamer in TE
- Optimized assay eliminated background signal in NTC



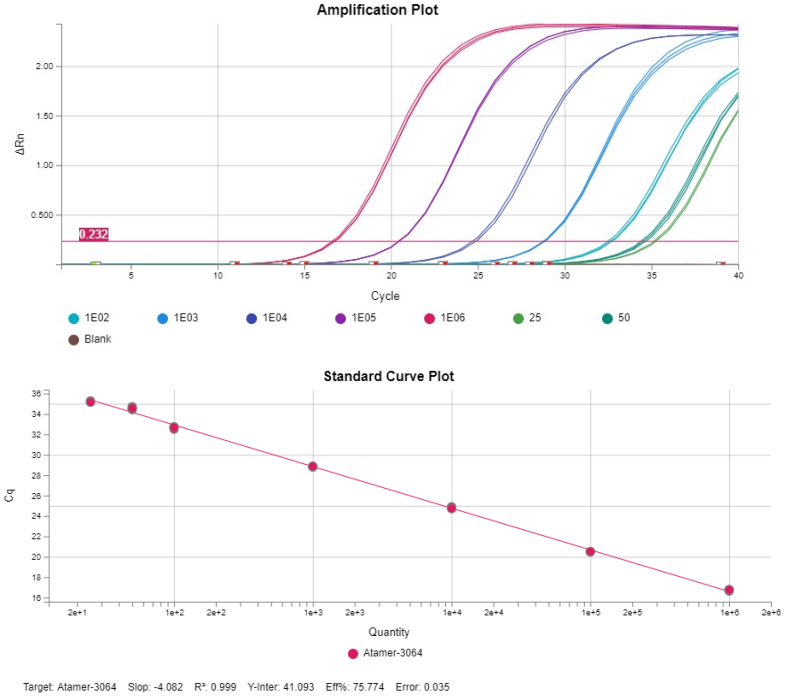
Direct detection of 39 nt aptamer in diluted plasma

- Serial dilution of 39 nucleotide aptamer in 5% plasma
- Used 5 µl per reaction for final plasma concentrations of 1.25%
- Assay conditions still under optimization
- Slopes of standard curves remain similar to aptamer in TE
- Signal in background wells still present with thresholds >35 cycles



Optimized detection of 40 nt aptamer in diluted plasma

- Serial dilution of 40nt nucleotide aptamer in 5% plasma
- Used 5 µl per reaction for final plasma concentrations of 1.25%
- Linear quantification down to 500 copies/reaction
  - Detection of amplification of 250 and 50 copies/reaction
- Slopes of standard curves remain similar to aptamer in TE
- Optimized assay eliminated background signal in NTC



Summary Assay Characteristics

Aptamer	Slope	y-intercept	R^2	Efficiency
39nt Aptamer	-3.52	38.7	0.999	92.2
40nt Aptamer	-4.09 & -4.16	41.1-41.3	0.997 & 0.999	74.0-75.6
50nt Aptamer	-3.48	35.6	0.999	93.8
51nt Aptamer	3.40 & -3.53	35.2-36.9	0.995 & 0.999	92.0 & 97.0
63nt Aptamer	-3.57	36.0	1.00	90.7
64nt Aptamer	-3.66	36.9	1.00	87.7

Conclusion

- Quantitative PCR a feasible approach for detection and quantification of DNA aptamers
  - Successful amplification of of 6 short, synthetic DNA aptamers of 39-64 nucleotides in length with SYBR green detection in QPCR assays
- Established optimized assay conditions for 40, 63, and 64 nucleotide aptamers
  - 51 nucleotide aptamer no amplification under any conditions: Re-design assay
  - 39 and 50 nucleotide aptamers with signal in NTC likely due to primer heterodimerization: Re-design assays
- Optimized assay sensitivity 50-500 total copies per reaction
- Successful amplification of aptamers (39-mer, 63-mer, and 64-mer) directly in canine plasma diluted to 5-10% plasma
  - Linear signal down to 100 copies/µl
  - Reduces time and costs associated optimization of sample preparation
  - Increases sample throughput in optimized assays
- With a LLOQ of 500 copies/reaction, a 1:20 dilution of plasma (5% plasma in input sample) would translate to a lower limit of 2,000,000 copies of aptamer/ml of plasma
  - LLOQ of GLP, validated dual-hybridization assay used for pegaptanib is $6*10^9$ copies/mL of plasma

Acknowledgments

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